Interleukin-6 induces Alzheimer-type phosphorylation of tau protein by deregulating the cdk5/p35 pathway

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Abstract

Inflammation is a process that has been actively related with the onset of several neurodegenerative disorders including Alzheimer disease (AD). However, the precise implications of inflammatory response for neurodegeneration have not been elucidated. A current hypothesis considers that extracellular insults to neurons could trigger the production of inflammatory cytokines by astrocytes and microglia. These cytokines, namely, interleukin (IL)-1β, TNFα, and IL-6, could affect the normal behavior of neuronal cells. In the present study, we describe the effect of the administration at physiologic doses of one of these cytokines, IL-6, to hippocampal neurons, on the protein kinase pathways as well as on the tau phosphorylation patterns. IL-6-treated neurons exhibited an increase in the amount of anomalously hyperphosphorylated tau protein in epitopes dependent on proline-directed protein kinases (PDPKs). On the basis of our data, the observed increase of tau epitopes of Alzheimer type is explained by an increase of intraneuronal levels of p35 activator and in the activity of the protein kinase cdk5 in response to this cytokine. Further confirmation of cdk5 involvement in this process was based on the findings that inhibition of the kinase activity with butyrolactone-I prevents the appearance of tau of Alzheimer type in IL-6-treated neurons. Additional studies suggest that an increase of cdk5 activity could be mediated by a known signaling cascade described for IL-6 function, namely, the MAPK–p38 signaling pathway. Stimulation of the IL-6 pathway appears to increase the tau epitopes of Alzheimer type, as demonstrated in studies with specific inhibitors. These results support the findings of a pathologic role for IL-6 in the neuroinflammatory response as related with the pathogenesis of neuronal degeneration.

Keywords: Interleukin-6; Cytokines; Alzheimer disease; cdk5/p35; Tau protein; MAPK–p38

Introduction

Interleukin-6 (IL-6) is a pleiotropic inflammatory cytokine secreted by lymphocyte cells in acute infection [1]. In the nervous system, IL-6 production has been shown to mainly occur in activated glia such as astrocytes and microglia cells [2]. IL-6 has been involved in the ethiopathology of neurodegenerative, as well as acute and chronic inflammatory diseases such as Alzheimer disease (AD) [3–5], Parkinson disease [6], systemic lupus erythematosus, multiple sclerosis, and HIV encephalopathy [7]. Under physiologic conditions, IL-6 and the IL-6 receptor (IL-6R) are expressed in several brain regions of the rat brain, including the hippocampus, striatum, hypothalamus, neocortex, and brainstem [8], and their expression is developmentally regulated [9].

In spite of these studies, the precise role of cytokines in neurodegeneration processes has not been fully understood. Thus, it has been described that cytokines secreted by microglia cells, astrocytes, and/or neuronal cells may induce synthesis of certain acute-phase proteins including the amyloid precursor protein AβPP [10,11]. On the other hand, AβPP or the amyloid-β peptide (Aβ) itself can induce the expression of IL-1β, TNF-α, and IL-6 in astrocytes and microglia cells in culture [12–17]. IL-6 has been found in the early stage of senile plaque formation [14,17]. In addition, IL-6 secretion by peripheral blood mononuclear

Abbreviations: IL-6, interleukin-6; PDPKs, proline-dependent protein kinases; Aβ, amyloid-beta peptide; IL-6R, IL-6 receptor.

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cells was increased in patients with AD as opposed to normal subjects, or those suffering from other brain disorder such as vascular dementia [18]. In addition to these observations, overexpression of IL-6 in the brain of transgenic mice that overproduce this cytokine [8] has been associated with a variety of neuropathologic findings including gliosis and selective disruption of cholinergic neurotransmission in the hippocampus [19]. Thus, a direct correlation has been established between the Aβ-induced neurotoxicity in neurodegenerative conditions, and cytokine production and its subsequent release.

A key role for inflammation in the ethiopathogenesis of AD has been proposed, but there is no clear correlation between the up-regulation of proinflammatory cytokines and tau hyperphosphorylation, one of the hallmarks of Alzheimer disease [20]. However, this particular role was observed for IL-1β in studies carried out in Alzheimer brains. IL-1β activates MAPK–p38 in vitro and is markedly overexpressed in Alzheimer brains [21]. On the other hand, we have shown that one of the main protein kinases involved in tau hyperphosphorylation in neurodegenerative disorders is the cdk5/p35 complex [22,23]. The cdk5/p35 complex plays a pivotal role in the development of nervous system as it has been shown through engineered targeted mutations of *cdk5*, *p35*, and *p39* genes producing cortical migration defects [24,25]. The function of this enzyme, a member of the proline-directed protein kinases (PDPKs) family that also includes the glycogen synthase kinase 3β (gsk3β), is regulated by the neurospecific activators p35 [26,27] and p39 [28]. Both kinases have been implicated in the ethiopathogenesis of Alzheimer disease [22,29]. However, changes on cdk5 regulation by varying the neuronal specific activator p35 and/or its soluble cytosolic fragment p25 have been correlated with an increase of tau phosphorylated epitopes of Alzheimer type in vitro and in vivo [22,26,28,30–32]. We have shown that Aβ peptide induces a marked increase of cdk5 activity paralleled by abnormal hyperphosphorylation of tau protein on hippocampal cells [20,22,23]. Moreover, the extracellular receptor kinase (ERK) and p38 factors phosphorylate tau protein in epitopes Ser^{202} and Thr^{205} [21], as well as the cdk5/p35 system. Besides, IL-6 can activate the JAK/STATs and the MAPK–p38 protein kinases; the latter is also involved in hyperphosphorylation of tau [33]. In this regard, Aβ-induced hyperphosphorylation of tau was decreased in presence of the specific inhibitor of MEK-1, PD98059 [33], an event also found in neuroblastoma cells exposed to the same inhibitor [33,34]. In the present study, we analyze the links between acute inflammation processes by using IL-6 as stimulatory signal over the cdk5 kinase system, a determinant of abnormally hyperphosphorylated tau of the Alzheimer type. An activation of cdk5/p35 system concomitant with tau hyperphosphorylations at Alzheimer epitopes was observed in hippocampal cells treated with IL-6.

Materials and methods

Reagents

Human recombinant IL-6 (Calbiochem, La Jolla) was dissolved in physiologic saline at 100,000 U/ml as stock solution and stored at −20°C. IL-6 was supplemented at a concentration of 5 ng/ml (500 U) to 4-day cultured hippocampal neurons that continued in culture for additional 48 h. Butyrolactone I, PD98059, SB203580, AG490, and JAK-3 were also obtained from Calbiochem and administered to cell cultures following manufacturer recommendations.

Cell cultures

Hippocampi were prepared from Sprague–Dawley rats. At embryonic day 18, animals were dissected, and primary rat hippocampal cultures were prepared as described [35]. Hippocampal cells were seeded into polylysine-coated coverslips for immunocytochemical analyses or into 35-mm plastic Petri dishes for biochemical experiments, and maintained in MEM supplemented with N2 (Gibco BRL) [36] plus streptomycin and penicillin. After four DIV of culture, the cells were treated with IL-6 (5 ng/ml) for 48 h.

Cell viability assay

The cells were seeded in polylysine-coated 96-well plates at 2.0×10^4 cells/100 μl per well in B27/MEM medium without phenol red. Then, cells were treated with increasing concentrations of IL-6 or PBS saline as a vehicle. After 48 h of incubation, cell viability was assayed by the modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay [23].

Immunoprecipitation and cdk5 activity assays

Hippocampal neurons were plated at 1×10^6 cells/cm^2 on polylysine-coated 35-mm dishes. Cultured neurons were exposed to IL-6 in N2/MEM medium for 48 h. Afterward, cells were lysed in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2 μg/ml aprotinin, 2 μM leupeptin, and 1 μg/ml pepstatin). Four hundred micrograms of cellular protein was immunoprecipitated with an anti-cdk5 antibody (C8 antibody; Santa Cruz Biotechnology), used at a final dilution of 1:50. For in vitro kinase assay, the immunoprecipitates were rinsed three times with RIPA buffer and one time with kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) plus1 μM cold ATP. The rinsed agarose beads were incubated with kinase buffer containing 2.5 μg of histone H1, plus 5 μCi [γ- ^{32}P] ATP in a final volume of 50 μl for 30 min at 30°C [23].

Primary antibodies

The following primary antibodies were used in this study: AT8, monoclonal antibody (mAb) against phosphorylated tau (Innogenetics, Belgium), Tau-1 mAb against dephosphorylated (kindly donated by Dr. Lester Binder, Northwestern University, IL), Tau-5 mAb against the conformational tau epitopes (donated by Dr. Lester Binder, Northwestern University, IL), polyclonal antibody against p35 (N20; Santa Cruz Biotechnology), polyclonal antibody p35 (C20, Santa Cruz Biotechnology), mAb against cdk5 (J3; Santa Cruz Biotechnology), polyclonal antibody ERK (Santa Cruz Biotechnology), mAb ERK-P (Santa Cruz Biotechnology), and polyclonal antibody Egr-1 (C-588, Santa Cruz Biotechnology). Actin mAb was from Sigma.

Immunodetection assays

The immunologic reactivity of the hippocampal cell extracts treated with cytokines and the untreated controls was assayed by Western blot techniques using antibodies that recognize Alzheimer phosphoepitopes on tau (AT8), and with the antibodies Tau-1 and Tau-5. The neuronal extracts from IL-6-treated neurons and controls were prepared according to the following steps. The cells were washed with cold PBS solution twice and scraped with a rubber policeman. Cells were homogenized, and the extracts were dissolved in buffer RIPA plus protease inhibitors: leupeptin (2 μg/ml), pepstatin (2 μg/ml), aprotinin (1 μg/ml), and PMSF (50 μg/ml). The samples were quantified by the Bradford protein assay using bovine serum albumin as a standard (Bio-Rad), then dissolved in Laemmli SDS-sample buffer, heat-denatured, reduced by addition of 5% β-mercaptoethanol, and electrophoresed on 10% SDS–PAGE minigels. The electrophoresis-separated proteins were then transferred by electroblotting onto nitrocellulose filters for 1 h at 100 V. Protein loading for all the lanes in nitrocellulose was determined by staining the membranes with the reversible dye Ponceau S. After blocking nonspecific sites on the membrane by incubation with 5% low-fat milk, expression of hyperphosphorylated proteins was determined by immunoblotting using AT8, Tau-1, and Tau-5 as antitau primary antibodies and using the ECL detection system (Amersham). Quantification of blots was carried out by scanning the photographic films of nitrocellulose membranes by using the Kodak digital Science densitometry program from Kodak. Statistical analyses were performed by the Sigma Plot software from Jandel.

Total RNA extraction and RT-PCR

Total RNA was extracted from control and IL-6-treated hippocampal cell cultures by using the TRIZOL (Gibco BRL) reagent. The amount of total RNA was quantified by spectrophotometric measurements at 260 nm. For RT-PCR experiments, 3 μg of total RNA was amplified in the presence of 100 ng of random primers in a final volume of 12 μl. Samples were then incubated for 10 min at 70°C followed by the addition of 4 μl of 5X transcription buffer, 2 μl of 0.1 mM DTT, 1 μl of 10 mM dNTP, and 200 U of Superscript II (Gibco BRL). The mixture was incubated at 42°C for 50 min in a final volume of 20 μl. For PCR amplification, different amounts of cDNA were analyzed to evaluate the linearity of the reaction. Subsequently, PCR amplification of samples was performed with specific primers against p35 and actin as internal control. Amplification was carried out with an initial denaturation of 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 90 s. A final step of extension was performed at 72°C for 3 min. PCR products were then analyzed in 2% agarose gel stained with ethidium bromide. P35 primers were forward: 5′ GACGCGTATGGGCACGGTGCTGTCC 3′, reverse: 5′ GCGCTAGCGGCTCACCGATCCAGCC 3′; and actin, forward 5′ TCTACAATGAGCTGCGTGTG 3′ and reverse 5′ TACATGGCTGGGGTGTTGAA 3′.

Results

IL-6 does not affect cell viability of the rat hippocampal neurons

Our first goal was to analyze the effects of the direct administration of IL-6 on the viability of neuronal cell cultures. Thus, we incubated rat hippocampal cells with increasing concentration of IL-6 ranging between 2 and 100 ng/ml, and then we quantified the extent of cell viability by using the MTT reduction assay. As shown in Fig. 1, the MTT assay indicates that between 10% and 12% of neurons died when cultured for 48 h in the presence of IL-6 at concentrations ranging from 2 to 10 ng/ml. However, an increase in IL-6 concentrations up to 40 ng/ml was deleterious for neuronal survival as indicated by a significant increase in cell death to around 23%; at 100 ng/ml IL-6, an extent of cell death of 35% was observed. These results are in close agreement with cell viability upon IL-6 treatment observed in other cell types [5]. Thus, on the basis of this study and to assure a high viability of neurons to allow observations on the detailed molecular aspects of the effects of IL-6 on hippocampal neurons, we developed most of the following experiments setting the cytokine concentration at 5 ng/ml. Our studies indicate that this IL-6 concentration was not deleterious to neuronal cells. It is worth observing that this IL-6 concentration represents half of the IL-6 concentration reported for an acute inflammatory response in the nervous tissue, and it corresponds approximately to the glial physiologic production of the cytokine. As a reference, under pathologic conditions, levels as high as 100 ng/ml have been observed in the cerebrospinal fluid of Alzheimer disease patients [6,18].

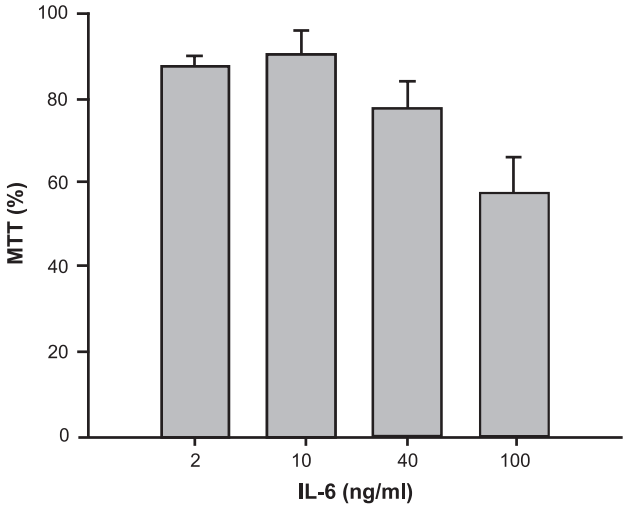

Fig. 1. Interleukin-6 (IL-6) effect on cells viability of rat hippocampal neurons. Cell viability was evaluated using the MTT reduction assay. Several groups of hippocampal cells were incubated with increasing concentrations of IL-6 for 48 h and the extent of cellular death analyzed. In the graph, each histogram represents the average of five experiments carried out under the same experimental conditions. The standard deviations are shown for each case. Hippocampal neurons incubated in absence of IL-6 were used as control. Further details are indicated in Materials and methods.

IL-6 induces an Alzheimer-type phosphorylation of tau in rat hippocampal neurons

Inflammation has been described to participate in neurodegeneration processes occurring in several degenerative disorders such as Alzheimer disease. However, little is known about the mechanism by which inflammation could contribute to neuronal degeneration. Thus, we analyzed the effects of IL-6 on tau hyperphosphorylation, a canonical hallmark for Alzheimer disease [20]. The exposure of hippocampal neurons to 5 ng/ml IL-6 increased tau epitopes of Alzheimer type as revealed by the levels of hyperphosphorylated tau recognized with the AT8 antibody (Fig. 2A, upper left panel). The AT8 epitopes were clearly diminished in control hippocampal cells treated under the same conditions but in the absence of IL-6 (Fig. 2A, upper left panel). The increase in the immunoreaction with AT8 was significant as indicated by the quantitative analyses ($n = 5$, $P < 0.01$) (Fig. 2A, right panel). Differences in the amount of phospho-dependent tau epitopes were not indeed due to changes of tau protein expression. An antibody that recognizes a conformational epitope on tau in a phosphorylation-independent manner (Tau-5) was used as an internal control

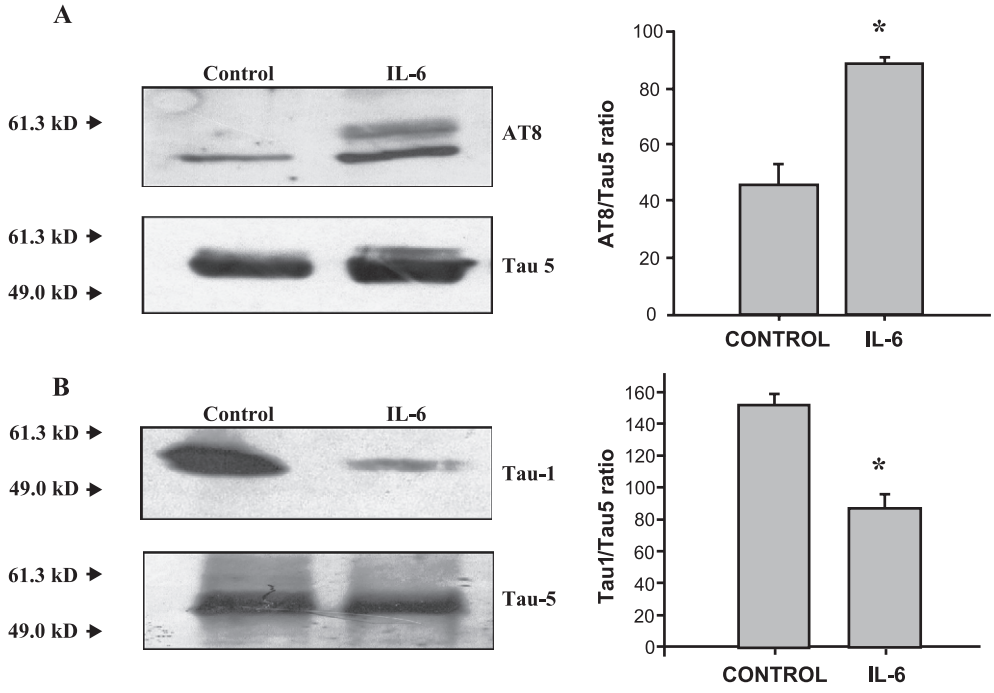

Fig. 2. Interleukin-6 (IL-6) induces tau hyperphosphorylation of Alzheimer type in embryonic rat brain hippocampal neurons. The treatment of hippocampal neurons with IL-6 (5 ng/ml) during 48 h increased the phosphorylation levels of tau protein, as immunodetected with the AT8 antibody. (A) Western blots of IL-6-treated hippocampal neurons (IL-6) and untreated controls (control), showing that IL-6 produces an increase in AT8 tau epitopes of Alzheimer type. No significant changes in tau epitopes were observed with Tau-5 antibody. In the right panel, we show a densitometric analysis from five different blot experiments with the ratio between phosphorylated tau (AT8) and total tau levels (Tau-5). The asterisk denotes significant differences in the IL-6-treated samples. (B) The increase in the AT8 epitopes (as shown in A) was paralleled by a decrease in the unphosphorylated tau epitopes as detected by Tau-1 antibody, with no variation in tau overall levels as indicated with Tau-5 antibody. In the right panel, we show a densitometric analysis from five experiments with the ratio between unphosphorylated tau (Tau-1) and total tau levels (Tau-5). The asterisk denotes significant differences in the IL-6-treated samples. Other details are indicated in Materials and methods.

(Fig. 2A, lower left panel), indicating no changes in the amount of total tau in the IL-6-treated neurons. Additionally, the increase in AT8 epitopes of tau was paralleled by a decrease in the unphosphorylated form of tau as evidenced by using the Tau-1 antibody (Fig. 2B, upper left panel). Tau-1 antibody recognizes tau with unphosphorylated epitopes that are susceptible of hyperphosphorylation by cdk5 and gsk3β protein kinases under conditions that favor neuronal degeneration. The decrease in Tau-1 reactivity in IL-6-treated cells was also significant as indicated in the quantification analyses ($n = 5$, $P < 0.05$) (Fig. 2B, right panel). Taken together, all these results indicate that the treatment of neurons with IL-6 induces tau hyperphosphorylation, with no additional effects on tau protein synthesis. Additionally, these changes were verified with no dramatic compromise of cell's viability (around 90% of cells were viable under these experimental conditions).

IL-6 induces an increase in the cdk5/p35 complex activity in rat hippocampal neurons

On the basis of previous observation, it was of importance to evaluate the activity of cdk5 in the IL-6-treated cell and untreated controls. The use of antibodies that tag the hyperphosphorylated tau epitopes such as AT8 and antibodies to the unphosphorylated forms such as Tau-1 allows us to

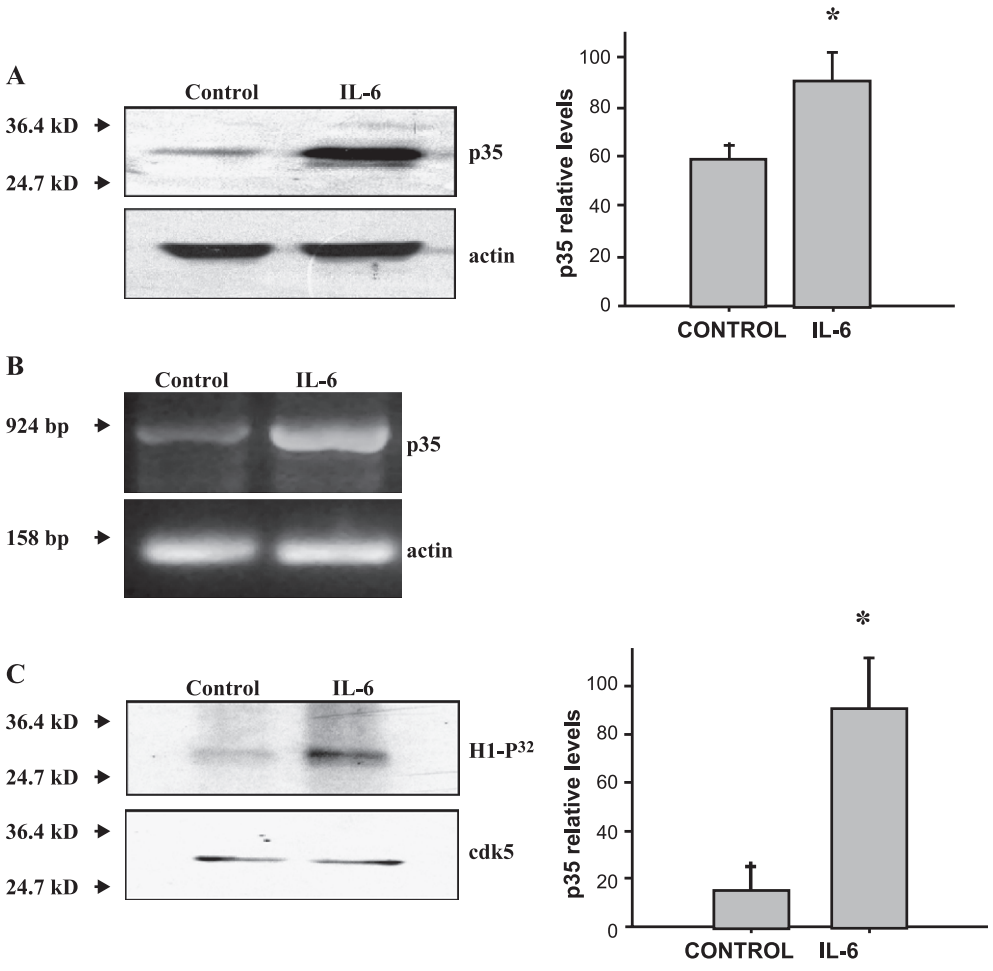

Fig. 3. IL-6 induced an increase in the cdk5/p35 complex activity in rat hippocampal neurons. IL-6 treatment of hippocampal neurons for 48 h increases the p35 protein levels as analyzed by immunoblotting and RT-PCR. Concomitantly, an increase in the activity of the kinase was verified. (A) Western blots of IL-6-treated hippocampal neurons (IL-6) and untreated controls analyzed with the N-19 polyclonal anti-p35 antibody, indicating an increase in the amount of the activator. Protein levels were normalized with the endogenous actin levels. In the right panel, we show the densitometric analysis from five different experiments, indicating significant differences in the amount of p35 in both control and IL-6-treated groups. (B) RT-PCR of IL-6-treated hippocampal neurons (IL-6) and untreated controls. IL-6 induced an increase in the DNA fragment of 924 bp corresponding to p35 as compared with untreated control. RT-PCR amplification was normalized with endogenous β-actin transcript. (C) The cdk5 kinase activity in hippocampal neurons exposed to IL-6 versus control cells (control) was determined after immunoprecipitation of cell homogenates (200 μg/sample) followed by incubation with ATP-P^{32} in the presence of calf thymus histone H1 as a substrate (upper panel). In both control and IL-6-treated cells, the amount of precipitated enzyme was equivalent, as indicated in the immunoblot (lower panel). In the right panel, we show the densitometric analysis from three independent experiments, indicating the increase of cdk5 activity normalized against immunoprecipitated enzyme. The asterisk represents significant differences between control and IL-6-treated groups. Bars in the right panel in A, B, and C represent standard deviations.

visualize tau phosphorylation patterns by PDPKs, making cdk5 a reasonable candidate for such an effect. Thus, the expression levels and enzymatic activity of the cdk5 system were then analyzed in IL-6-treated neurons. Fig. 3 shows the changes in the expression pattern of the cdk5 activator p35 in response to such treatments. IL-6 promoted a significant increase in the levels of the neurospecific cdk5 activator, p35, as recognized with the specific N-20 antibody ($n = 5$, $P < 0.05$) (Fig. 3A, left and right panels). This increase in p35 levels could be due to an increase in the transcriptional control of p35 activator. To substantiate this possibility, we performed RT-PCR from control and IL-6-treated neurons. IL-6 treatment induced a significant increase of a 924-bp DNA fragment corresponding to p35 (Fig. 3B). Actin was used in both Western blot and RT-PCR experiments as an internal control, with no significant variations between control and treated groups. It has been proposed that the soluble proteolytic fragment of p35, called p25, may be also responsible for tau hyperphosphorylation in Alzheimer disease [31]. We analyzed the amount of p25 in IL-6-treated neurons and found no increase of this proteolytic fragment (data not shown). Besides p35 expression, we evaluated the cdk5 activity. Studies indicated that IL-6 treatment of hippocampal neurons increased the activity levels of the complex cdk5/p35 about five times as compared with untreated controls, as corroborated by densitometric analysis ($n = 3$) (Fig. 3C, upper left and right panels). A Western blot with anti-cdk5 was used as an internal control in the activity experiments showing that cdk5 levels were the same in both groups in the activity assays (Fig. 3C, lower panel). The results suggest that the overexpression of activator p35 (Figs. 3A and B) may contribute to the increase of cdk5 activity and the subsequent tau phosphorylation in the hippocampal cells treated with IL-6, although a cdk5 activation by a molecular mechanism not elucidated (possibly posttranslational modifications, etc.) may also be involved in the increase of the enzyme activity.

IL-6-induced tau hyperphosphorylation is dependent on cdk5/p35 complex

The increase on tau hyperphosphorylation triggered by IL-6 was suggested to be dependent on cdk5, since p35 activator was increased in response to IL-6, and therefore the cdk5/p35 activity complex was amplified. cdk5 and gsk3β can catalyze tau phosphorylation of Alzheimer-type epitopes recognized by AT8 antibody. We then analyzed the contribution of cdk5 for such phosphorylation increase. IL-6 neurons were treated with 5 μM butyrolactone-I, an inhibitor that, at the concentrations indicated, blocks cdk5 activity rather than the other cdk's in the hippocampal cell model [23]. As shown in Fig. 4A (upper left panel), the increase in AT8 tau phosphorylation induced by IL-6 treatment was

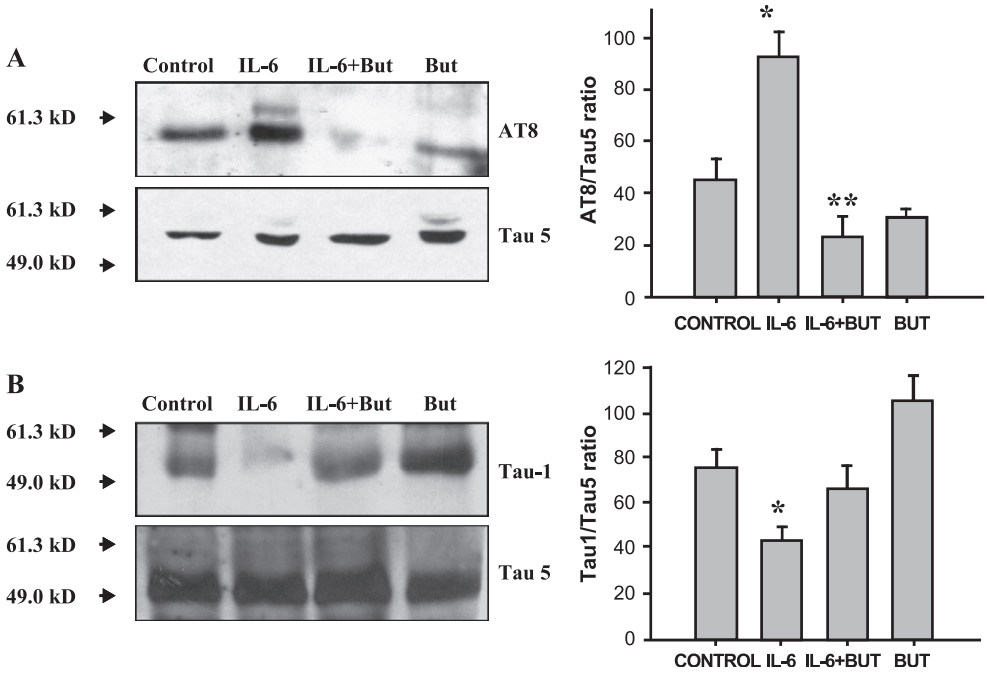

Fig. 4. Hyperphosphorylation of tau protein by IL-6 is dependent on cdk5/p35 complex. The increase in the hyperphosphorylation of tau protein induced by IL-6 was abolished by butyrolactone-1, a specific inhibitor of cdk5. (A) Western blot analysis of IL-6-treated hippocampal neurons during 48 h in presence of butyrolactone-1 (But) shows a severe decline in IL-6-induced tau hyperphosphorylation, as detected by immunoreaction with AT8 antibody. The right panel shows a densitometric analysis from three experiments normalized with respect to the total amount of tau, immunodetected with Tau-5 antibody (asterisks denote significant differences). (B) Western blot analysis of IL-6-treated hippocampal neurons during 48 h in presence of butyrolactone-1 (But) showing a severe decline in the IL-6-induced tau hyperphosphorylation, as detected by immunoreaction with Tau-1 antibody. Below are Western blots stained with Tau-5 for total tau. The right panel shows a densitometric analysis from three different experiments normalized by the total amount of tau protein.

abolished in the presence of butirolactone-I. The inhibition of cdk5 led to a decrease in the phosphorylated tau to levels even below the untreated cells controls. No variations were found with the phosphorylation-independent antibody to tau, Tau-5 (Fig. 4A, lower left panel). Quantification of these data is shown in the right panel. Concomitantly, studies with the Tau-1 antibody gave complementary results (Fig. 4B, upper left and right panels). Tau-1 epitopes are decreased after IL-6 treatment. This decrease was reverted in the presence of a cdk5 inhibitor as shown in Fig. 4B, attaining levels above the controls.

Therefore, in our hippocampal culture, system inhibition of cdk5 with butyrolactone-I produced a dramatic decrease in AT8 immunoreactivity, indicating a blockage on tau phosphorylation, with no substantial changes for tau protein levels as shown with Tau-5 antibody (Fig. 4B, lower panel). Altogether, these results implicate cdk5 as a kinase involved in tau hyperphosphorylation in response to IL-6.

IL-6 activates the JAK/STATs and the MAPK pathways in hippocampal neurons

Our next step was to investigate the molecular mechanisms that could be controlling such an increase of p35 protein and the cdk5/p35 activity complex. The effects of cytokines are mainly transduced by a signaling pathway involving the JAK/STAT activation [7] and by MAPK-dependent signaling [1,7,37]. Thus, our research was devoted to analyze the activation of the JAK/STATs cascade in the IL-6-treated hippocampal neurons. To test the effect of this signaling pathway, we decided to measure the levels of both activated phospho-STAT3 (p-STAT3) recognized with an antibody against the phosphorylated factor and phospho-ERKs kinase in response to IL-6 treatments. Fig. 5 shows that IL-6 treatment induced an increase in both activated forms phospho-ERK1/2 (Fig. 5A, upper panel) and phospho-STAT3 (Fig. 5B). The

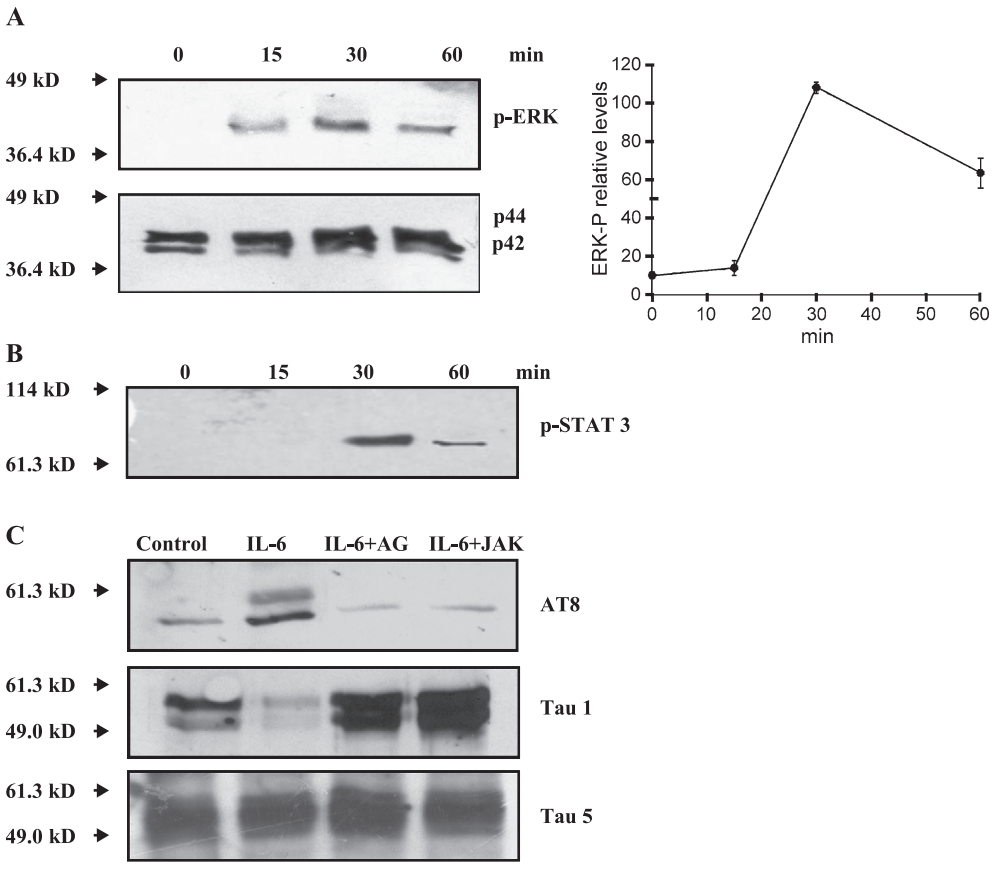

Fig. 5. Activation of JAK/STATs and the MAPK pathway in response to IL-6 treatment in hippocampal neurons. (A) Western blots of IL-6-treated hippocampal neurons (IL-6) for 15, 30, and 60 min analyzed with anti-phospho-ERK antibody (activated ERK forms). Below, another set of treated and untreated cells was analyzed as an internal control with an anti-ERK antibody that recognizes total activated and nonactivated ERK. The plot indicates the time course of ERK activation, as measured by p-ERK levels detected. Each point represents three different experiments with their respective standard errors. (B) Western blots of IL-6-treated hippocampal neurons (IL-6) for 15, 30, and 60 min analyzed with anti-phospho-STAT3 antibody. The activation of ERKs pathway is correlated with the activation of STAT3 factor in IL-6-treated hippocampal neurons. (C) Western blots of untreated controls (control) and IL-6-treated hippocampal neurons (IL-6), in the presence of specific inhibitors to JAK2 activation, AG 490 (10 μM) (IL-6 + AG) or JAK3 activation, JAKIII inhibitor (30 μM) (IL-6 + JAK), showing that kinase inhibitors induce a severe decline in the IL-6-promoted tau hyperphosphorylation, as detected by immunoreaction with the AT8 antibody. The increase in the AT8 epitopes (as shown in C) was paralleled by a decrease in the unphosphorylated tau epitopes as detected by Tau-1 antibody, with no variation in tau overall levels as indicated with Tau-5 antibody.

time-dependent increase of the activated phospho-ERK1/2 did not reflect necessarily an increase in the total pool of ERK kinase, as indicated in an internal control using an antibody that recognizes ERK protein isoforms p44 and p42 in a phosphorylation-independent manner (Fig. 5A, blots in the lower panel). Therefore, a plot of time course of ERK activation (p-ERK formation) indicates an increase in the activation process after 15 min. A peak in the levels of both phopho-STAT3 and phospho-ERK1/2 was observed at 30 min of IL-6 treatment, data that are in agreement with previous reports in different cell types, that is, hippocampal neurons, brain astrocytes, and in lymphocytes [7]. Given the evidence that IL-6 was indeed activating ERK and JAK/STAT signaling pathways, we decided to test whether inhibition of these cascades could have an effect upon tau hyperphosphorylation. In one set of experiments, we used combined treatments of IL-6 along with 100 nM AG490 and 10 μM JAK-3, two inhibitors of the JAK/STAT pathway. JAK-3, inhibited JAK phosphorylation induced by IL-6, whereas AG490 inhibited JAK autophosphorylation occurring previously to JAK effect upon STAT phosphorylation. Notably, both compounds were able to reverse tau hyperphosphorylation as denoted with AT-8 antibody (Fig. 5C, upper panel). Consistently, both inhibitors induced an increase of unphosphorylated tau as denoted by Tau-1 antibody after IL-6 treatment as expected (Fig. 5C, middle panel). All of the aforementioned changes occurred with no variations in the total amount of tau protein (Fig. 5C, lower panel). Altogether, these results suggest that part of the effects produced by IL-6 in our system was transduced by the JAK/STAT signaling pathway leading to an increase in tau phosphorylation.

IL-6-induced hyperphosphorylation of tau is a MAPK–p38 dependent pathway

The MAPK–p38 pathway has been involved in the hyperphosphorylation of tau protein [21,34]. Furthermore, this signal transductions pathway has been reported to be activated in hippocampal neurons in response to IL-6 treatment [1,3,5]. Therefore, we investigated the MAPK–p38 contribution to the IL-6-induced hyperphosphorylation of tau protein. Mammalian p38 has been shown to be activated through cellular stress. Cell surface receptors involve the activation of specific protein kinases in an ordered activation module [38].

Thus, we decided to analyze the involvement of MAPK–p38 pathway in the hyperphosphorylation of tau protein after IL-6 treatment. As indicated in Fig. 6, IL-6-dependent tau hyperphosphorylation was significantly reduced in the presence of the p38 inhibitor SB203580 (SB) at a concentration of 20 μM (Fig. 6A, upper panel). This inhibition of tau hyperphosphorylation, as detected with AT8 antibody, is not due to changes in the levels of the protein as confirmed by using the Tau-5 antibody (Fig. 6A, lower panel). Interestingly, the inhibition of p38 by SB reduced the IL-6-promoted phosphorylation of the slower migrating tau isoform to the levels of the controls. Changes in the phosphorylation pattern of the faster tau isoforms were less dramatic, although the inhibitor also decreased phosphorylation of those tau isoforms (Fig. 6A, upper panel). Tau hyperphosphorylation inhibition by 30 μM PD98059 (PD), a MAPK inhibitor, was observed. The incubation with PD significantly reduced the slower tau isoform, representative of tau phosphorylation of Alzheimer type (Fig. 6A, upper panel), in agreement with data reported in Aβ-treated neurons exposed to PD [33]. Densitometry analysis (Fig. 6B) confirmed the significance of differences among IL-6-treated cells with respect to control (*), IL-6 with respect to IL-6 + SB (**), and the significance of difference between IL-6-treated cells with respect to IL-6 + PD (***). These results indicate that MAPK–p38 pathway appears to be involved in the IL-6-induced tau hyperphosphorylation. The relationship with cdk5 is analyzed below.

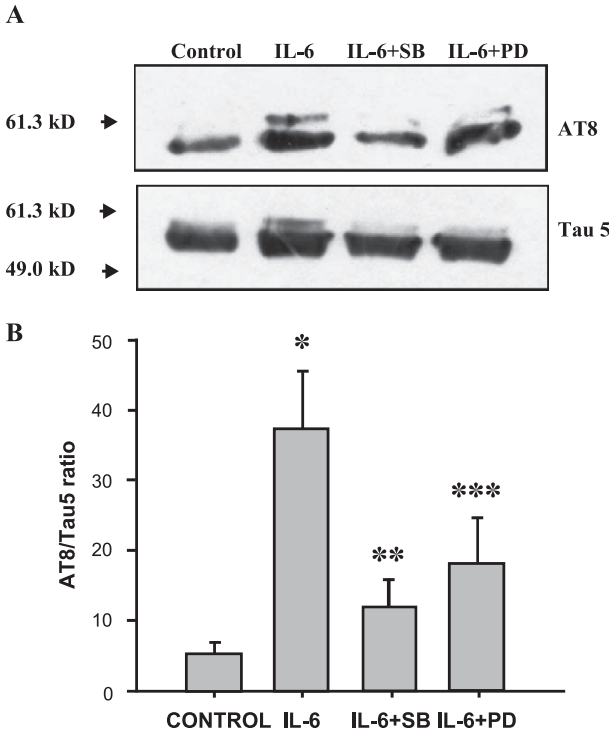

Fig. 6. Role of MAPK–p38 pathway in the hyperphosphorylation of tau protein induced by IL-6. The inhibition of MAPK–p38 pathway decreased the hyperphosphorylation of tau protein by IL-6. (A) Western blot analysis of IL-6-treated hippocampal neurons during 48 h in the presence of MAPK-specific inhibitor PD98059 (IL-6 + PD) or the inhibitor of p38 SB203580 (IL-6 + SB) shows that kinase inhibitors induce a partial decline in the IL-6-promoted tau hyperphosphorylation, as detected by immunoreaction with the AT8 antibody. This decrease in tau phosphorylation is clearly evidenced in the slow-migrating band of around 60 kDa in the blot. In the lower panel, the immunodetection with Tau-5 antibody is shown. Below is a densitometric analysis from three independent experiments of data from Western blot analyses, normalized by the total amount of tau protein.

IL-6-increased p35 levels diminished in the presence of MAPK–p38 pathway specific inhibitors

The relative increase in p35 levels induced by IL-6 appears to be sensitive to the pharmacologic inhibition of the MAPK–p38 pathway. The treatment with 20 μM SB and 30 μM PD diminished the IL-6-stimulated p35 protein levels in hippocampal neurons. As seen in Fig. 7, the levels of p35 in the IL-6-treated neurons in the presence of 30 μM PD were significantly lower than those of IL-6-treated cells without the inhibitor (*$P < 0.05$) and were comparable with those of p35 controls ($n = 3$). Moreover, the p38 inhibitor, SB, partially reduced the p35 protein levels overstimulated by the IL-6 treatment ($n = 3$). Quantification of data reflects these observations, indicating a major effect of PD inhibitor as compared with SD. To test whether activation of MAPK–p38 pathway could be able to activate cdk5, we performed cdk5 activity assays in the presence of SB and PD inhibitors. As shown in Fig. 7B, both compounds were able to reverse the increase of cdk5 activity induced after IL-6 treatment, suggesting that MAPK–p38 pathway could act upstream of cdk5 function. The net effect of this signaling pathway may be a more active cdk5/p35 complex due to increased levels of p35.

IL-6 increased the Egr-1 protein levels in hippocampal neurons. Role of the Egr-1 factor in the p35 increased level by IL-6 treatment

The p35 levels can be modulated by stimulation of the transcription factor Egr-1 as a result of preactivation of the MAPK pathway in cortical neurons [39]. Egr-1 is a transcription factor involved in the expression of many genes involved in stress response [40], differentiation [41], and growth of many cellular types [42]. This factor can be induced by stimulation of different proinflammatory cytokines such as TNF-α [42]. Moreover, it was shown that p38-C-junk pathway regulates the Egr-1 levels in epithelial cells [40,43]. On the basis of this information, we decided to evaluate the Egr-1 protein levels in the IL-6-treated neurons, considering the possible relationship between MAPK pathway activation and the p35 increased levels found in IL-6-treated hippocampal neurons. In Fig. 8, we show that IL-6 treatment increased Egr-1 protein levels

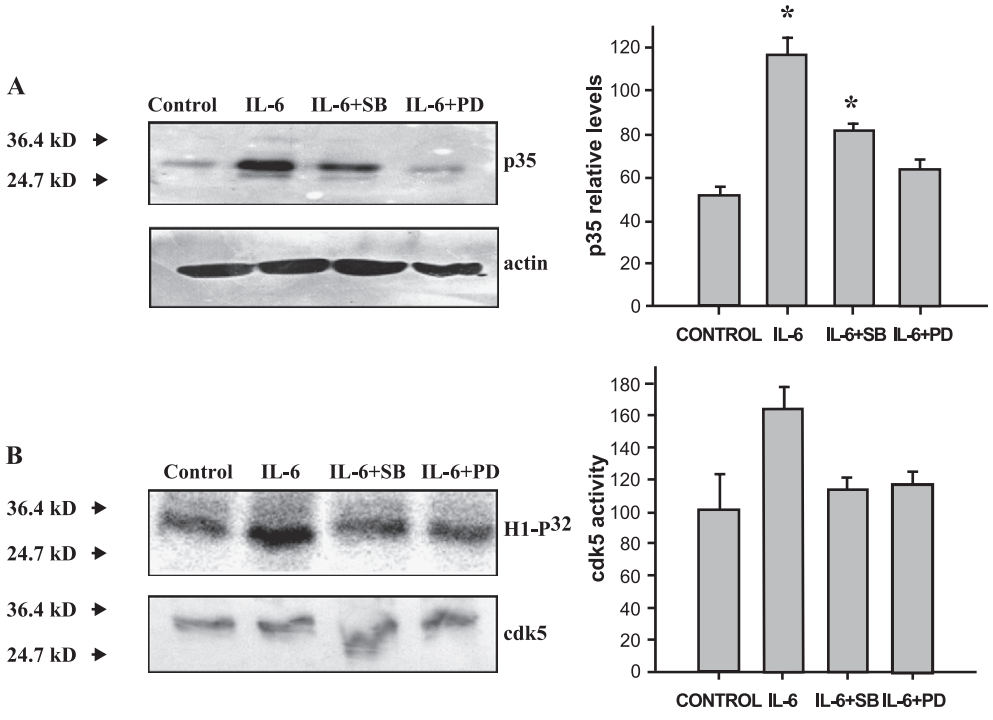

Fig. 7. IL-6-increased levels of p35 were diminished in presence of MAPK–p38 pathway-specific inhibitors. The increase in the p35 levels induced by IL-6 is sensitive to the pharmacologic inhibition of MAPK–p38 pathway. (A) Western blot of untreated control (control) and IL-6-treated hippocampal neurons, in the presence of either the p38-specific inhibitor pathway SB3089 (20 μM) (IL-6 + SB), and MAPK-specific inhibitor PD98059 (30 μM) (IL-6 + PD) during 48 h. The figure shows the decrease in the IL-6-stimulated levels of p35 by action of PD. The incubation with SB induced a partial decrease in the p35 level stimulated by the cytokine treatment. Actin was used as an internal control in both treated and untreated cells. In the right panel, we show a densitometric analysis from three independent experiments. (B) The cdk5 kinase activity in IL-6-treated hippocampal neurons during 48 h in the presence of MAPK-specific inhibitor PD98059 (IL-6 + PD) or the inhibitor of p38 SB203580 (IL-6 + SB) shows that kinase inhibitors induce a decline in the IL-6-promoted increase cdk5 activity, as detected by immunoprecipitation of cell homogenates (200 μg/sample), followed by incubation with ATP-P^{32} in the presence of calf thymus histone H1 as a substrate (B, upper panel). In all conditions, the amount of precipitated cdk5 was equivalent, as indicated in the immunoblot (lower panel). In the right panel, we show the densitometric analysis from three independent experiments, indicating the increase of cdk5 activity normalized against immunoprecipitated enzyme. Bars in the right panel in A and B represent standard deviations.

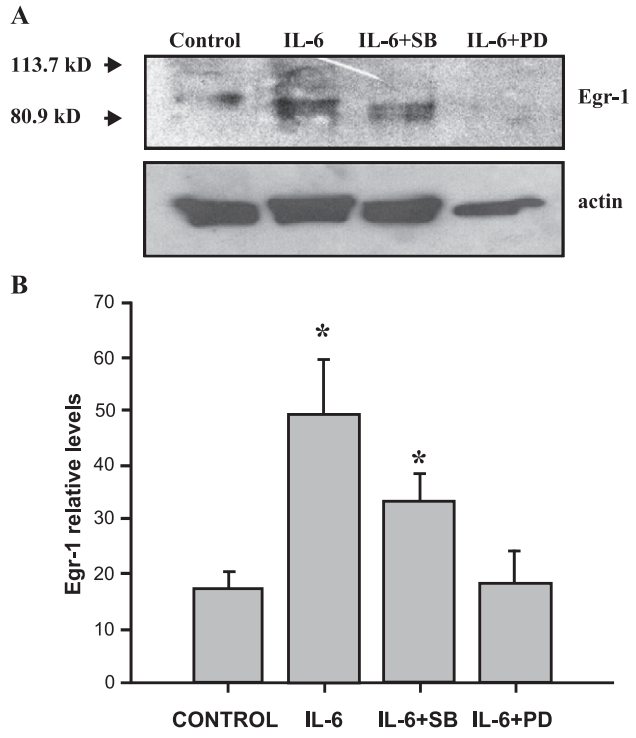

Fig. 8. IL-6 induced an Egr-1 increase in rat hippocampal neurons. The treatment of hippocampal neurons with IL-6 (5 ng/ml) during 48 h increased the Egr-1 levels, as immunodetected with the anti-Egr-1 (C-588) polyclonal antibody. (A) Western blots of untreated controls (control) and IL-6-treated hippocampal neurons (IL-6), in the presence of specific inhibitors to p38 pathway, SB3089 (20 μM) (IL-6 + SB) or MAPK pathway, PD98059 (30 μM) (IL-6 + PD), showing that IL-6 produces an increase in the Egr-1 levels, which is prevented by the MAPK inhibitor. The incubation with SB decreased the Egr-1-stimulated levels, but to a lower extent. Actin was used as an internal control in both treated and untreated cells. (B) Densitometric analysis from three different experiments, indicating that Egr-1 relative levels normalized against endogenous actin levels. Asterisks indicate significant differences between control and IL-6 with or without p38/MAPK inhibitors.

detected by the anti-Egr-1 polyclonal antibody C-588. Here, we can observe the expression of the 80-kDa protein band, in agreement with previous reports in lymphocytes and neuroblastoma cells [39,44]. Quantification of Western blot data based on three different experiments support the strong inhibitory effect of PD on Egr-1 expression (Fig. 8B). This increase in Egr-1 levels is paralleled with an increased in the p35 mRNA as indicated in Fig. 3B, suggesting that an increase of Egr-1 may result in increased transcriptional activity that ultimately controls p35 levels. Thus, our studies showed that the pharmacologic inhibition of MAPK−p38 pathway by PD decreased Egr-1 levels stimulated by IL-6. Previous studies showed that the Egr-1 levels could be modulated by a partial block of MAPK and p38 pathways [39,44]. Therefore, our data on the study of IL-6-induced neuronal degeneration in hippocampal cells appears to be well supported by other studies. Altogether, these results indicate that IL-6 changes p35 levels by activation of MAPK pathway. This activation could be regulated by the Egr-1 levels with the subsequent effects in p35 expression.

Discussion

In this report, we have described the effect of IL-6 on hippocampal cells in primary cultures. Survival of neurons treated with 5 ng/ml of IL-6 was not compromised, thus allowing to carry most of our studies under conditions that assure a high cell viability. One of the most significant effects of IL-6 on hippocampal neurons analyzed in this study was an increase in hyperphosphorylated tau of Alzheimer type, as denoted by an IL-6-induced increase in the phospho-specific epitopes recognized by AT8 and PHF1 antibodies. Such kind of effects for IL-6 has not been reported previously and suggests a possible role in the neurodegenerative pathway of neurons. These changes in tau phosphorylation patterns are most likely to be dependent on cdk5 [23], since the activity of cdk5/p35 complex as well as the levels of a neuron specific cdk5 activator, p35, markedly increased in response to the cytokine treatment. Furthermore, in support of these findings, the cdk5 inhibitor butyrolactone-I prevented IL-6-promoted cdk5 activation and tau hyperphosphorylation.

The significant increase of both phosphorylated STAT3 and ERK1/2 kinases suggests the participation of canonical IL-6 signaling pathway, dependent on MAPK effect, in the activation of cdk5 in IL-6-treated neurons. Other interesting findings indicated that IL-6 treatment induced a consistent increase in the p35 levels. This effect could be connected with the Egr-1 increment in the IL-6-treated neurons, since concomitantly with Egr-1 increased levels (Fig. 8), we observed an increase in both p35 mRNA and protein levels (Figs. 3A and B). Certainly, the increase of p35 in response to Egr-1 induction is consistent with previous findings suggesting MAPK control over cdk5 pathway [44]. These are very important observations since we are showing that IL-6 increases the p35 activator level, an increment which is sensitive to MAPK specific inhibitors, and that Egr-1 levels are elevated in response to the treatment with this proinflammatory cytokine. Thus, the mechanisms responsible for IL-6-induced tau hyperphosphorylation could involve an activation of MAPK signaling pathway followed by an increase in Egr-1 expression and elevated p35 levels. This effect seems to be strongly linked with the three- to fourfold increase in the cdk5/p35 complex activity as triggered by the IL-6 treatment.

On the other hand, it has been previously described that activation of ERKs and p38 could lead to tau hyperphosphorylation [45–47]. Although we cannot rule out a direct action of MAPK and p38 upon tau phosphorylation, the complete inhibition of tau hyperphosphorylation induced by IL-6 in cells treated with the cdk5 inhibitor suggests that MAPK and p38 could play a role in tau phosphorylation induced by IL-6 through cdk5 action.

The inflammatory response has been suggested to play a critical role in the ethiopathogenesis of Alzheimer disease. High levels of proinflammatory cytokines, such as IL-6, IL1β, and TNF-α, have been detected in AD patients [4,6,14] and in other brain pathologies [48]. On the other hand, IL-6R is present in both immature and adult neurons, although its function remains elusive [49]. Moreover, it has been reported that oxidative stress, that is, the one produced by amyloid-β peptide, can induce IL-6 production and release from astrocytes in vitro [12,17,37] and in vivo [15,47], thus contributing to neuroinflammatory response. Although inflammation has been actively related with the onset of AD [3–6,14,17], little is known about the mechanisms underlying the IL-6 effects and its contribution to the neurodegeneration. Reactive astrocytosis is a typical hallmark of neurodegeneration found in several studies conducted to reproduce AD both in vitro and in vivo [29,50]. A role for inflammatory response in neurodegeneration was also depicted in a study dealing with the overexpression of IL-6 in the astrocytes of a transgenic mice model [8,19]. Further studies with this mutant mouse showed learning impairment [8,19], abnormal nervous system physiology [19], and memory defects [8,19], supporting a role for IL-6-induced neurodegeneration. However, the participation of IL-6 in the generation of pathologic hallmarks of AD still remains to be elucidated, and our study contributes to this analysis from the point of view of tau hyperphosphorylation analysis.

In this report, we show for the first time that a protein kinase known to be deregulated in AD [22,23] is involved in the transduction mechanisms induced by IL-6. Neurons stimulated with different concentrations of the cytokine showed a consistent elevation in the levels of hyperphosphorylated tau. This increase in the amount of Alzheimer-type tau was paralleled with a decrease in the unphosphorylated form of the protein as indicated with Tau-1 antibody. The generation of Alzheimer-type epitopes induced by IL-6 was prevented by pharmacologic inhibition of the cdk5 kinase and increased the cdk5/p35 complex activity around three to four times. Thus, it seems reasonable to think that IL-6 effects on neurons are transduced by a signaling pathway involving the cdk5/p35 complex.

A prominent feature of Alzheimer disease is an abundance of activated glia in close proximity of senile plaques. These activated astrocytes and microglia can participate in the propagation of inflammatory and oxidative stress response [51]. The mechanisms underlying astrogliosis and free radical production have been shown to be related with the MAPK signaling pathway [52], as well as by mechanisms that control the intracellular levels of the divalent cation, calcium [53–55]. However, no data has been available on the molecular route leading to tau hyperphosphorylation in IL-6-treated degenerating neurons. IL-6 activates the JAK/STAT complex and subsequently the MAPK pathway. In this study, we showed an increase of both phosphorylated-STAT3 and ERK1/2 in response to IL-6 treatment. Recently, it has been proposed that by changing the activity of Egr-1 transcription factor through phosphorylation, MAPK signaling pathway could control the amount of p35 activator and, in that way, the activity of the cdk5 complex [39,44]. Thus, the changes in STAT3 and ERK phosphorylation patterns and the increase of p35 levels described in this report clearly support a mechanism dependent on MAPK signaling pathway acting upstream of cdk5. In addition, IL-6 has been shown to modify the intracellular calcium levels on neurons. Such modifications depend on a calcium influx mediated by NMDA activation by IL-6 rather than a release of the cation from intracellular stores and calcium influx through voltage-dependent channels [53]. This increase on intracellular calcium levels has been proposed to affect cdk5 activity. Thus, calcium elevation should be responsible for the enhanced calpain activity leading to increased proteolysis of p35 activator, to give rise the more soluble and more stable p25 fragment [31]. The increase of p25 fragment in pathologic conditions such as AD and its role in cdk5 promoting activity is somewhat more controversial [31,56,57]. In our study, we were unable to detect measurable changes in the amount of p25 fragment, suggesting that cdk5 activation due to IL-6 should not require p35 proteolysis.

Previous work of our laboratory implicated to cdk5 deregulation in the increase of Alzheimer-type hyperphosphorylated tau [22,23]. Moreover, these studies suggested the role of cdk5 deregulation in neurodegeneration induced by amyloid-β peptide [26]. Amyloid-β is known to produce oxidative stress that could trigger inflammatory response in glial cells in vitro [10,47] and in vivo [15]. Part of the aforementioned response should consider the production and secretion of inflammatory cytokines in response to Aβ peptide, thus promoting the neurodegenerative cascade of events leading to tau hyperphosphorylation inside neurons. Additionally, a genetic component involved in the IL-6 gene regulation could also be related with the onset of AD [58,59]. Altogether, the present data indicate that IL-6 is directly involved in the up-regulation of the cdk5/p35 activity complex and suggest that inflammation could contribute by already known mechanisms for IL-6 function to the appearance of hyperphosphorylated tau in neuron, a molecular hallmark for Alzheimer disease.

Acknowledgments

The authors thank Dr. Lester Binder for his generous gift of Tau-1 and Tau-5 antibodies and Dr. Peter Davis for the valuable PHF1 antibody. This research was supported by grants from FONDECYT grant 1020155 to R.B.M., FONDECYT grant 3010060 to C.G.-B., and the Millennium Institute for Advanced Studies (CBB), project P99-031F from the Millennium Science Initiative.

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